POLYEPITOPE VACCINES

1. This application is a continuation of co-pending application Serial No. 08/776,337 filed April 21, 1997, which is a nationalization under 35 U.S.C. §371 of International Application number PCT/AU95/00461 filed July 27, 1995, which claims priority to Australian Patent Application number PN1009 filed February 8, 1995, and Australian Patent Application number PM7079 filed July 27, 1994.

BACKGROUND OF THE INVENTION

1. Field of the Invention

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2. The present invention relates to vaccines containing a plurality of cytotoxic T lymphocyte epitopes and to polynucleotides including sequences encoding a plurality of cytotoxic T lymphocyte epitopes.

2. Description of the Related Art

3. CD8 + αβ cytotoxic T lymphocytes (CTL) recognize short peptides (epitopes, usually 8-10 amino acids long) associated with specific alleles of the class I major histocompatability complex[1] (MHC). The peptide epitopes are mainly generated from cytostolic proteins by proteolysis, a process believed to involve the multicatalytic proteosome complex[2-7]. Peptides of appropriate length are transported into the endoplasmic reticulum where specific epitopes associate with MHC. The MHC/epitope complex is then transported to the cell surface for recognition by CTL. The influence of sequences flanking CTL epitopes on the proteolytic processing of these epitopes remains controversial[8-12]. However, by constructing a recombinant vaccinia coding for an artificial polypeptide protein containing nine CD8 CTL epitopes in sequence, the present inventors have determined that the natural flanking sequences of CTL epitopes are not required for class I processing, that is each epitope within the polyepitope protein was always efficiently processed and presented to appropriate CTL clones by autologous polyepitope vaccinia infected target cells.

Summary of the Invention

4. Accordingly, in a first aspect, the present invention consists in a recombinant polyepitope cytotoxic T lymphocyte vaccine, the vaccine comprising at least one recombinant protein including a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, wherein the at least one recombinant protein is substantially free of sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

5. Preferably, the at least one recombinant protein does not include any sequences naturally found to flank the cytotoxic T lymphocyte epitopes. However, it should be understood that small lengths (e.g. 1-5 amino acids) of sequences naturally found to flank the cytotoxic T lymphocyte epitopes may be included. The phrase "substantially free of sequences naturally found to flank the cytotoxic T lymphocyte epitopes" is to be taken as including such short lengths of flanking sequences.

6. In a second aspect, the present invention consists in a polynucleotide, the polynucleotide including at least one sequence encoding a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, and wherein the at least one sequence is substantially free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

7. Again, it is to be understood that "substantially free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes" includes the possibility of including short peptide (e.g 1-5 amino acids) sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

8. In a third aspect, the present invention consists in a nucleic acid vaccine, the vaccine comprising the polynucleotide of the second aspect of the present invention and an acceptable carrier.

9. In a fourth aspect, the present invention consists in a vaccine formulation, the vaccine comprising the recombinant protein of the first aspect of the present invention and an acceptable carrier and/or adjuvant.

10. In a preferred embodiment of the present invention the at least one recombinant protein includes, and the at least one sequence encodes, at least three cytotoxic T lymphocyte epitopes.

11. In a further preferred embodiment, the epitopes are from multiple pathogens.

Brief Description of the Drawings

18. FIG. 1. Construction of a recombinant vaccinia that expresses a synthetic DNA insert coding for the polytope protein (SEQ ID NO:10), which contains nine CTL epitopes in sequence. Boxed sequences representing linear B cell epitopes.

19. FIG. 2. CTL recognition of each epitope expressed in the recombinant polytope vaccinia construct of FIG. 1.

20. FIG. 3. Polytope vaccinia can recall epitope specific responses. Bulk effectors from donors (A) CM - A24, A11, B7, B44; (B) YW - A2, B8, B38 and (C) NB - A2, A24, B7, B35 were generated by infecting peripheral blood mononuclear cells (PBMC) with the polytope vaccinia of FIG. 1., SEQ ID NO: 10, at a MOI of 0.01 for 2 hours followed by 2 washes. After 10 days culture in 10% FCS/1640 RPMI the bulk effectors were used against autologous phytohaemagglutinin T cell blasts target cells (E:T 20:1) sensitized with the indicated peptide (10μM) in a standard 5 hour chromium release assay[14]. Peptides used were (A) SEQ ID NO: 6, SEQ ID NO: 4, SEQ ID NO: 9, SEQ ID NO: 8; (B) SEQ ID NO: 6, SEQ ID NO: 11, SEQ ID NO: 4, SEQ ID NO: 17; and (C) SEQ ID NO: 6 and SEQ ID NO: 21. Bulk effectors generated by the addition of irradiated autologous A type LCLs[14] (LCL to PBMC ratio 1:50) gave similar results to that shown above.

21. FIG. 4. Construction of a polytope DNA insert including ten murine CTL epitopes as in Table 2.

22. FIG. 5. Polypeptide sequence (SEQ ID NO: 22) of the polytope DNA insert (SEQ ID NO: 23) of FIG. 4.

23. FIG. 6A. Results of CTL assays conducted on splenocytes harvested from BALB/c mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 19.

24. FIG. 6B. Results of CTL assays conducted on splenocytes harvested from BALB/c mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 18.

25. FIG. 6C. Results of CTL assays conducted on splenocytes harvested from BALB/c mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 20.

26. FIG. 6D. Results of CTL assays conducted on splenocytes harvested from BALB/c mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 12.

27. FIG. 6E. Results of CTL assays conducted on splenocytes harvested from CBA mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 13.

28. FIG. 6F. Results of CTL assays conducted on splenocytes harvested from CBA mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 3.

29. FIG. 6G. Results of CTL assays conducted on splenocytes harvested from C57BL/6 mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 1.

30. FIG. 6H. Results of CTL assays conducted on splenocytes harvested from C57BL/6 mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 14.

31. FIG. 6I. Results of CTL assays conducted on splenocytes harvested from C57BL/6 mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 15.

32. FIG. 6J. Results of CTL assays conducted on splenocytes harvested from C57BL/6 mice vaccinated with recombinant vaccinia including the DNA insert of FIG. 5 (open

squares), no peptide control (open triangles), or bulk splenocytes from TK-vaccinated mice (open circles) and restimulated with the peptide of SEQ ID NO: 5.

33. FIG. 7. Comparison of spleen MCMV titres (± standard error) 5 weeks after polytope vaccinia vaccination and 4 days after MCMV challenge. P values - unpaired Student's t-test

34. FIG. 8. DNA vaccination with different plasmids in BALB/c mice.

35. FIGS. 9-28. Lysis of target cells presenting 10 different epitopes by splenocytes from murine polytope vaccinia immunized (IP) mice. Splenocytes from strains BALB/c, (FIGS. 9-20), and C56BL/6 (FIGS. 21-28) had the spleens removed and splenocytes restimulated with the following peptides: FIGS. 9 and 10, SEQ ID NO: 19; FIGS. 11 and 12, SEQ ID NO: 18; FIGS. 13 and 14, SEQ ID NO: 20; FIGS. 15 and 16, SEQ ID NO:12; FIGS. 17 and 18, SEQ ID NO:13; FIGS. 19 and 20, SEQ ID NO:3; FIGS. 21 and 22, SEQ ID NO: 1; FIGS. 23 and 24, SEQ ID NO: 14; FIGS. 25 and 26, SEQ ID NO:16; FIGS. 27 and 28, SEQ ID NO:5. The effectors were then used in standard CTL assays against peptide-coated target cells, using the same peptide (squares) or no peptide controls (circles in FIGS. 9, 11, 13, 15, 17, 19, 21, 23, 25, and 27), against virus-infected targets (FIGS. 10, 12, 14, 16, 18, 20, 22, 24 and 28), or against tumor targets (FIG. 26). Virus infected targets were either infected with allantoic fluid as negative control (FIGS. 10 and 18), with human polytope vaccinia (Vacc Nu PT) as the negative control (FIGS. 12, 14, 16, 20, 22, 24 and 28), or the EL-4 line served as a control (FIG. 26).

Description of Preferred Embodiments

Example 1

36. Nine, class I restricted, CTL epitopes from several Epstein-Barr virus nuclear antigens (EBNA) have previously been defined using CTL clones[10, 18-20]. The clones were isolated from a panel of normal healthy Epstein-Barr virus (EBV) seropositive donors and were restricted by different HLA alleles (Table 1). A recombinant polyepitope vaccinia (polytope vaccinia), which coded for a single artificial protein containing all nine of these CTL epitopes, was constructed (see FIG. 1). The DNA sequence coding for this protein was made using splicing by overlap extension (SOEing) and the polymerase chain reaction (PCR) to join six

overlapping oligonucleotides. The insert was cloned into pBluescript II, checked by sequencing and transferred into pBCBO7[15] behind a vaccinia promoter to generate pSTPT1. This plasmid was then used to generate the polytope vaccinia virus by marker-rescue recombination[16]. The artificial polytope protein expressed by this vaccinia therefore containing no sequences naturally found to flank the CTL epitopes in their proteins of origin (FIG. 1).

CTL CLONES	COGNATE EPITOPES	SEQ ID NO:	SOURCE	HLA RESTRICTION	REFS
LC13	FLRGRAYGL	6	EBNA3	B8	13
LC15	QAKWRLQTL	11	EBNA3	B8	14
CS31	EENLLDFVRF	4	EBNA6	B44	15
YW22	SVRDRLARL	17	EBNA3	A0203	14
CM4	KEHVIQNAF	9	EBNA6	B44	13
NB26	YPLHEQHGM	21	EBNA3	B3501	14
LX1*	HLAAQGMAY	7	EBNA3	?	14
JSA2	DTPLIPLTIF	2	EBNA3	B51#/A2	13
CM9	IVTDFSVIK	8	EBNA4	A11	16

Table 1: Description of the CTL clones, their cognate epitopes, the proteins of origin (source) and their HLA restriction. The first two letters of the clones refer to the donors. *Not tested (see FIG. 2). #Recent evidence suggests this epitope may be restricted by HLA-B51. All the epitopes have been minimalized except EENLLDFVRF (SEQ ID NO: 4) and DTPLIPLTIF (SEQ ID NO: 2).

37. A DNA sequence coding for the polytope amino acid sequence was designed with codons most frequently used in mammals and incorporated a Kozac sequence[13] and a BamHI site upstream of the start codon. Six 70mer oligonucleotides overlapping by 20 base pairs were spliced together using Splicing by Overlap Extension (SOEing)[14] in 20μl reactions containing standard PCR buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 1.5U of Taq polymerase (hot start at 94°C) using the following thermal program: 94°C for 10 seconds, 45°C for 20 seconds and 72°C for 20 seconds (40 cycles). Half of each gel purified dimer sample was combined in a second PCR reaction (12 cycles) with the addition of 0.5μl of α^{32}p dCTP. The reaction was run on a 6% acrylamide gel and a slice corresponding to the position of the hexamer product was isolated. Two 20mer oligonucleotides were used to PCR amplify the hexamer using an annealing temperature of 56°C and 25 cycles. The gel purified fragment was cloned into the EcoRV site of pBluescript II KS-, was checked by sequencing and cloned behind the vaccinia P7.5 early/late promoter using the BamHI/SalI sites in the vaccinia plasmid vector pBCBO7[15] to generate pSTPT1. Construction of a TK- recombinant virus was carried out using marker rescue combination between pSTPT1 and VV-WR-L929 as described previously[16]. Plaque purified

virus was tested for TK phenotype and for appropriate genome arrangement by Southern blotting of viral DNA[16].

38. To establish whether each epitope could be efficiently processed from the polytope protein the polytope vaccinia was used to infect a panel of target cells, which expressed the HLA alleles restricting each epitope. Autologous CTL clones specific for each epitope were then used as effector cells in standard chromium release assays. In all cases tested the CTL clones recognized and killed the HLA matched target cell infected with the polytope vaccinia and the appropriate (see Table 1) EBNA vaccinia (positive controls), but not the TK-vaccinia (negative controls) (FIG. 2).

39. Figure 2 shows CTL recognition of each epitope expressed in the polytope vaccinia construct. Effector CTL are listed in Table 1 (E:T ratio 5:1). Target cells (see below) were infected with recombinant vaccinia expressing either (i) the EPV nuclear antigen (EBNA) recognized by the CTL clone (see Table 1) (positive control), (ii) TK- (negative control), or (iii) the polytope construct (i.e., Polytope vaccinia). Vaccinia infection of the target cells was at a multiplicity of infection of 5:1 followed by 14-16 hour incubation at 37°C prior to use in the standard, 5 hour, ^{51}Cr-release assay[29]. Clone LX1 was no longer available at the time of assay. Target cells; there are two types of EBV, A and B-type, whose EBNA protein sequences differ significantly. CTL clones LC13, LC15, CM4, NB26, JSA2 and CM9 recognize cells transformed with A-type EBV but not B-type EBV, and CTL clones CS31 and YW22 recognize cells transformed with A-type EBV and EBV[10, 18-20]. The target cells used for the A-type specific CTL were therefore autologous lymphoblastoid cell lines transformed with B-type virus (B-type LCLs). The target cell for CS31 and YW22 were EBV negative B cell blasts, established using anti-CD40 antibody and rIL-4[21].

40. An additional series of experiments used the polytope vaccinia to stimulate *in vitro* a secondary CTL response from peripheral blood mononuclear cells (PBMC) obtained from healthy EBV seropositive donors. The resulting bulk CTL cultures were then used as effectors against peptide epitope sensitized autologous PHA blasts in a standard chromium release assay. The polytope vaccinia was capable of recalling CTL responses which were specific for epitopes restricted by the HLA alleles expressed by each donor (FIG. 3).

41. Figure 3 shows that polytope vaccinia can recall epitope specific responses. Bulk effectors from donors (A) CM - A24, A11, B7, B44; (B) YW - A2, B8, B38 and (C) NB - A2,

A24, B7, B35 were generated by infecting peripheral blood mononuclear cells (PBMC) with the polytope vaccinia at a MOI of 0.01 for 2 hours followed by 2 washes. After 10 days culture in 10% FCS/1640 RPMI the bulk effectors were used against autologous phytohaemagglutinin T cell blasts target cells (E:T 20:1) sensitized with the indicated peptide (10μM) in a standard 5 hour chromium release assay[19]. Bulk effectors generated by the addition of irradiated autologous A type LCLs[19] (LCL to PBMC ratio 1:50) gave similar results to that shown above.

42. Two linear B cell epitopes (STNS, SEQ ID NO: 24 and NNLVSGPEH, SEQ ID NO: 25) recognized by monoclonal antibodies (8G10/48[22] and 8E7/55[23] respectively) were incorporated at each end of the polytope construct (FIG. 1) to follow the expression of the polytope protein. Western blotting and indirect immunofluorescence antibody staining of polytope vaccinia infected lymphoblastoid cell lines (LCLs) and the processing defective T2 cell line[6,7] using these antibodies failed to detect polytope protein products (data not shown). Recombinant proteins expressed by vaccinia using the same P7.5 promoter are usually readily detected[24] implying that the polytope protein was rapidly degraded in the cytoplasm of mammalian cells. This degradation was not dependent on LMP2 and 7 since the T2 cell line does not express these proteosome associated endopeptidases[6,7]. This phenomenon is consistent with other studies expressing truncated proteins or peptides in mammalian cells[25] and is likely to reflect the inability of such proteins to fold into any secondary or tertiary structures.

43. A glutathione S-transferase fusion expression vector containing the human polytope was constructed. The DNA fragment coding for the human polytope was excised from pBSpolytope using BamHI/HincII and cloned into the BamHI/AmaI restriction sites in pGex-3x (GST Gene Fusion System Pharmacia) to make pFuspoly. This plasmid was used to express the polytope fusion in bacteria using the standard induction protocols. An aliquot of the bacteria was lysed in loading buffer and run on a 20% SDS PAGE gel with size markers. This gel indicated that the expected protein of approximately 38kD (the human polytope plus the GST domain (26kD)) was being expressed in bacteria containing the plasmid. Western blotting with the two monoclonal antibodies 8G10/48 and 8E7/55 demonstrated that the fusion detected contained the human polytope which has the two linear B cell epitopes (STNS and NNLVSGPEH respectively) incorporated at each end of the polytope construct. This protein may be incorporated into liposomes or ISCOMs.

44. Attempts to purify the fusion protein using the GST purification employing glutathione agarose beads failed due to the lack of fusion protein in the bacterial extract supernatant. All the fusion protein precipitated with the cell debris. The protocol was not at fault since GST expressed by itself in a different bacterial culture was in the bacterial extract supernatant and could be purified easily. These data suggest the fusion protein is rapidly degraded in the bacteria unless sequestered into bacterial inclusion bodies from which purification using the GST system is difficult.

Example 2

MATERIALS AND METHODS

45. **Construction of a recombinant vaccinia expressing the murine polytope protein**. Ten class I murine CTL epitopes from various diseases were selected so that there were two epitopes for each of H-2Db, H-2Kb, H-2Kd, H-2Kk and H-2Ld which are represented in three strains of mice (see Table 2). These amino acid sequences were arranged such that each of the first 5 epitopes was restricted by a different HLA allele followed by the second group 6-10. The two groups of epitopes were converted to a DNA sequence using the universal codon usage data. These two DNA sequences were separated by an SpeI and flanked by a XbaI restriction site at the 5' end and a AvrII site at the 3' end. Also incorporated at the 5' end is a BamHI restriction site, a Kozac sequence[13] and a methionine start codon. While at the 3' end there is a B cell epitope from *Plasmodium falciparum*, a stop codon and a SalI restriction site see Figures 4 and 5. Five 75mer oligonucleotides and a 76mer oligonucleotide overlapping by 20 base pairs, representing this 341 base pair sequence, were spliced together using Splicing by Overlap Extension (SOEing)[14] and the polymerase chain reaction (PCR). Primer dimers were made of primers 1 and 2, 3 and 4, 5 and 6 (0.4μg of each) in 40μl reactions containing standard 1x *Pfu* PCR buffer, 0.2 mM dNTPs and 1U of Cloned *Pfu* DNA polymerase (hot start at 94°C) using a Perkin Elmer 9600 PCR machine programmed with the following thermal program; 94°C for 10 seconds, 42°C for 20 seconds and 72°C for 20 seconds for 5 cycles. At the end of 5 cycles the PCR program was paused at 72°C and 20μl aliquots of reactions 2 and 3 were mixed (reaction 1 was left in the PCR machine) and subjected to a further 5 cycles. At cycle 10 the program was paused again and 20μl of reaction 1 added to the combined reactions 2 and 3 and a further 5 cycles completed. The combined 40μl sample was then gel purified on a 4% Nusieve agarose gel (FMC) and a gel slice corresponding to the correct sized fragment removed and spun through

Whatmann 3MM paper. Two 20mer oligonucleotides were used to PCR amplify the full length product using the standard reaction mix as above and an annealing temperature of 50°C and 25 cycles. The full length PCR fragment was gel purified in a 4% Nusieve agarose gel, cloned into the EcoRV site of pBluescript IIKS$^-$ to make pBSMP and checked for mutations by sequencing. The DNA insert of a plasmid containing the correct sequence was excised using BamHI/SalI restriction enzymes and cloned, using the same enzymes, behind the vaccinia P7.5 early/late promoter in the plasmid shuttle vector pBCB07[15] to generate pSTMOUSEPOLY. Construction of a TK- recombinant virus was carried out using marker rescue recombination between pSTMOUSEPOLY and VV-WR-L929 using protocols described previously[16]. Plaque purified virus was tested for TK phenotype and for appropriate genome arrangement by Southern blotting of viral DNA[17].

46. **Vaccination of mice with recombinant murine polytope vaccinia.** The recombinant vaccinia was used to vaccinate 3 mice in each of the 3 strains of mice BALB/cv, C57BL/6 and CBA. The vaccinations were I.V. 50μl containing 5 x 10^7 pfu of vaccinia and the mice were left to recover for three weeks. The TK- vaccinia was used as a negative control for each strain of mouse in this experiment.

47. **Cytotoxic T cell assays.** Splenocytes were harvested from the vaccinated mice 3 weeks post vaccination and restimulated with the appropriate peptides (1μg/ml) in vitro[16]. No peptide were used for restimulations as negative controls. After 7 days of culture the restimulated bulk effectors were harvested and used in a 5 hour, ^{51}Cr-release assays. The targets used in these assays were ConA blasts generated from each of the strains coated with one of the peptides presented by that strain. Three effector to target ratios were used 50:1, 10:1 and 2:1 the results are shown in FIGS. 6A-6J.

RESULTS

Construction of murine recombinant polytope vaccinia,

48. The list of epitopes included in the murine polytope are listed in Table 2.

Table 2 CTL epitopes of the murine CTL polytope

SOURCE	SEQUENCE	SEQ ID NO:	RESTRIC-TION	MOUSE STRAIN
Influenza nuclear protein (366-374)	ASNENMDAM	1	$H\text{-}2D^b$	C57BL/6
Adenovirus 5 E1A (234-243)	SGPSNTPPEI	14	$H\text{-}2D^b$	C57BL/6
Ovalbumin (257-264)	SIINFEEKL	15	$H\text{-}2K^b$	C57BL/6
Sendai virus nuclear protein (324-332)	FAPGNYPAL	5	$H\text{-}2K^b$	C57BL/6
Influenza nuclear protein (147-155)	TYQRTRALV	19	$H\text{-}2K^d$	BALB/c
P. Berghei Circumsporozoite protein (249-257)	SYIPSAEKI	18	$H\text{-}SK^d$	BALB/c
Influenza nuclear protein (50-58)	SDYEGRLI	13	$H\text{-}2K^k$	CBA
Influenza NS1 (152-160)	EEGAIVGEI	3	$H\text{-}SK^k$	CBA
Murine Cytomegalovirus pp89 (168-176)	YPHFMPTNL	20	$H\text{-}2L^d$	BALB/c
Lymphocytic choriomeningitis virus nuclear protein (118-126)	RPQASGVYM	12	$H\text{-}SL^d$	BALB/c

49. The construction of the polytope DNA insert is summarized in FIG. 4. The polytope sequence is shown in Fig 5.

CTL assays.

50. Each epitope in the polytope induced a primary CTL response in mice with the appropriately MHC allele. No competition between two epitopes restricted by the same allele was observed. (the high flu NP response in CBA mice given TK- controls is likely to be due to a naturally acquired influenza).

51. Polytope constructs containing multiple CTL epitopes from various pathogens restricted by various MHC alleles are clearly capable of generating primary CTL responses to each epitope within the polytope vaccine. This has clear application in all vaccines where CTL responses are required for protection. For instance, multiple HIV CTL epitopes might be

combined in a therapeutic vaccine to foreshadow epitopes expressed by escape mutants and thereby prevent disease progression.

52. Murine polyepitope mice have SIINFEKL specific CTL which can kill the ovalbumin transfected cell line EG7 in vitro and in vivo.

SIINFEKL specific CTL which kill the EG7 tumor cells demonstrated in vitro

53. Spleen cells from murine vaccinia immunized mice were collected 4 weeks post vaccination and restimulated in vitro with 10ug/ml SIINFEKL for 7 days. Effectors could not lyse the untransfected parent line EL4 but could lyse the EG7 tumor cells and EL4 cells sensitized with SIINFEKL.

Protection against EG7 tumor in vivo afforded by murine polytope

54. Mice (C57B6) were given either human polytope vaccinia (Thomson et al., 1995) or murine polytope vaccinia (10^7 pfu/mouse/ip) and 4 weeks later received 10^7 EL4 or EG7 tumor cells (Moore et al., 1988. Cell 54,777) subcutaneously (10 or 11 mice per group).

55. The number of mice with visible tumors (all were >1cm diameter) at day 9 is given.

Human Polytope Vaccinia		Murine Polytope Vaccinia	
EG7	EL4	EG7	EL4
10/10*	10/10	0/11	10/10

*(Two mice had tumors <1cm in diameter)

Protection against MCMV

56. BALB/c mice were challenged with MCMV (K181 strain, 8 x 10^3 PFU, 100μl intraperitoneally) 5 weeks after polytope vaccinia vaccination. Four days after challenge the viral titres per gram of spleen were determined the results are shown in FIG.7 (method of Scalzo *et al*)[17].

Evaluation of polytope vaccines delivered in a DNA plasmid.

57. The polytope protein described above contained a linear antibody epitope recognized by a monoclonal antibody. As described above the polytope protein could not, however, be detected in cells infected with the polytope vaccinia indicating that it is very unstable; a likely consequence of having no folding structure. It was thus considered that

delivery of a polytope vaccine may be best achieved using nucleic acid vaccination technology or with an adjuvant system that protects from proteolysis (e.g. liposomes or ISCOMs).

58. The CMV promoter cassette from pCIS2.CXXNH (Eaton *et al* (1986) *Biochemistry* **25**(26) p8343) was cloned into the EcoRI site of pUC8 in the same orientation as the LacZ gene to make the plasmid pDNAVacc (used as a control plasmid in the DNA vaccination experiments). This plasmid then had the murine polytope (from pBSMP) inserted into the XhoI site in the multiple cloning site to form pSTMPDV. The plasmid pRSVGM/CMVMP has fragments sourced from a number of different plasmids. The RSV promoter was excised from pRSVHygro (Long *et al* (1991) *Hum. Immunol.* **31**, 229-235), the murine GM-CSF gene from pMPZen(GM-CSF) (Johnson *et al* (1989) *EMBO* **8**, 441-448) and the CMV promoter cassette from pCS (Kienzie *et al* (1992) *Arch. Virol.* **124** p123-132). Into the CMV cassette was the murine polytope cloned into the SmaI site of the multiple cloning site. Both genes , murine GM-CSF and the murine polytope, use the bi-directional polyA from SV40.

59. Nine 6 week old female BALB/c mice were injected I.M. with 50μg of either pDNAVacc (plasmid control), pSTMPDV (murine polytope only) or pRSVGM/CMVMP (murine GM-CSF and murine polytope) in 50μl of PBS (see next figure). They were given boosters with another 50μg of the same plasmids at 3 weeks. At 8 weeks from the vaccination these mice were killed and their spleens removed. Splenocytes were isolated and cultured with peptide as previously described for vaccinia vaccinated animals. These bulk effectors were then used in standard ^{51}Cr release assays against P815 cells coated with peptide corresponding to the epitopes in the murine polytope that are presented by BALB/c mice. The assay was done for 6 hours at E:T ratios of 2:1, 10:1 and 50:1.

60. The results of these experiments are shown in Fig 8.

SPECIFIC CTL ACTIVITY AGAINST PEPTIDE COATED AND VIRUS INFECTED TARGETS INDUCED BY THE MURINE POLYTOPE VACCINIA

Method

61. 1. **Vaccination and Effector Cell Preparation.** Mice (3 per group) were vaccinated intraperitoneally (IP) with 5 x 10^7 PFU vaccinia. Mice were boosted via the same route and with the same amount of vaccinia week 3. The spleens were removed 6 weeks after the initial vaccination and the splenocytes were isolated after erythrocyte lysis with ACK Buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA)(Current Protocols in Immunology, Ed JE

Coligan, AM Kruisbeek, DH Margulies, EM Shevach, W Strober, 1994 John Wiley and Sons Inc. USA.). 5 x 10^6 splenocytes per well were peptide restimulated (1µg/ml) in bulk T cell media (RPMI/10% Fetal Calf Serum (FCS), 2mM Glutamine, $5x10^{-5}$M 2-Mercaptoethanol) for seven days prior to cytotoxic T lymphocyte (CTL) assay on ^{51}chromium (^{51}Cr) labeled target cells[17]. The peptides used for restimulation are given above A to J. The effectors were used against either peptide coated targets A-J, viral infected targets (A'-J') or transfected antigen expressing targets (I').

62. **2. Preparation of Target Cells.** Cell lines used as targets in these assays were P815 for BALB/c (H-2^d), EL-4 and EG7 for C57BL/6 (H-2^b), L929 for CBA (H-2^k) L929, or con A blasts prepared from the BALB/c, C57BL/6 or CBA mice, respectively.[1] To express the required epitope for CTL killing, target cells were either pre-incubated with (i) peptide (A-J), (ii) vaccinia (B'-D', F'-J'). or (iii) Influenza (A', E'), or maintained as the (iv) Ovalbumin-expressing plasmid transfectant of El-4 (EG7) in the case of the SIINFEKL epitope system (I').

63. (i) Peptide coated targets (A-J): Target cells were centrifuged at 1000rpm/5 min. The supernatant was discarded to approximately 200µg/ml and 10-20µl of either RPMI (No peptide) or 200ug/ml stock peptide in RPMI (peptide coated) (final concentration 10µg/ml) was added to the cell pellet. One hundred microliters of ^{51}Cr was added to cell pellet and the cells were incubated at 37°C for 1 hr. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at 10^5/ml for target cells in the CTL assay.

64. (ii) Vaccinia (Vacc.) infected targets (B'-D', F'-J'): Vaccinia used for virus infected targets were the Murine Polytope (Vacc Mu PT), with the Human Polytope (Vacc Hu PT) as the negative control. Cell lines infected by vaccinia were P815 (B'-D'), L929 (F') and EL-4 (G'-J'). The target cells were centrifuged at 1000rpm/5 min. The supernatant was discarded to approximately 200ul and the cells (approx. 10^6 cells) infected with vaccinia at a multiplicity of infection (MOI) of 10:1 by adding 20µl vaccinia (10^9 pfu/ml) followed by incubation for 1 hr at 37°C. Five milliliters of RPMI/10%FCS was then added, cells mixed and incubated overnight at 37°C. These cells were subsequently centrifuged and supernatant discarded into camdyne. One hundred microliters of ^{51}Cr was added to cell pellet and the cells incubated at 37°C for 1 hr. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at 10^5/ml for target cells in the CTL assay.

65. (iii) Influenza infected targets (A', E'): The A/PR/8/34 strain of Influenza virus was used for the BALB/c targets (A') and the reassortant A/Taiwan/1/86 (IVR-40) for the CBA targets (E'). Allantoic fluid was used as the negative control. Cell lines infected by influenza were P815 (A') and L929 (E'). Target cells were centrifuged at 1000rpm/5 min. and supernatant was discarded. Five hundred microliters: 50μl Influenza virus (10^8/ml EID) or Allantoic Fluid, 50μl ^{51}Cr, 400μl RPMI/1%FCS was added to the cell pellet and incubated for 1 hr at 37°C. Ten milliliters of RPMI/10%FCS was added, mixed and incubated a further 2 hr at 37°C. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at 10^5/ml for target cells in CTL assay.

66. (iv) Ovalbumin expressing targets (I'): EG7 cells are EL-4 cells transfected with an expression plasmid containing chicken ovalbumin cDNA (Moore MW, Carbone FR and Bevan BJ (1988) Introduction of soluble protein into Class 1 pathway of antigen processing and presentation. *Cell* 54: 777-785.). These cells were maintained in RPMI/10% FCS. 20mM Hepes, 2mM Glutamine, 1mM Na Pyruvate, 1001U/ml penicillin and 100μg/ml Streptomycin. The plasmid was selected and maintained in Geneticin (G-418) at 500μg/ml once per month. EL-4 cells with no peptide (EL4 no pep) were used as the negative control. The cells were centrifuged at 1000rpm/5 min. and supernatant discarded to approximately 200μl. One hundred microliters of ^{51}Cr was added to cell pellet and the cells incubated at 37°C for 1 hr. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at 10^5/ml for target cells in the CTL assay.

67. **3. CTL Assay.** The restimulated splenocytes ($5x10^6$/ml) were dispensed (100μl) in triplicate at three Effector: Target ratios (50, 10, $2x10^4$ effector cells: $1x10^4$ target cells for the CTL assay. One hundred microliters of target cells (10^5/ml) were added to all effectors and control wells (Spontaneous release = 100μl media; Maximal release = 100μl 0.5% SDS/ RPMI/10%FCS). Microtitre plates were centrifuged at 500rpm for 5 min. and incubated at 37°C for 6hr. Plates were recentrifuged at 500rpm/5 min. and 25μl of supernatant was counted for ^{51}Cr release. Percentage Specific Lysis represents averages of triplicate counts: 100 x (Test cpm - Spontaneous cpm)/(Maximal cpm - Spontaneous cpm).

68. The results are shown in FIGS. 9-28.

DNA vaccination experiment

69. The initial DNA vaccination experiments illustrate that the polytope can be delivered using DNA vaccination. In addition, that vaccination may be improved by the co-delivery of a cytokine gene (GM-CSF), although in this experiment the improvement is not statistically significant.

70. The current system is clearly sub-optimal. Likely improvements would be the use of potentially better plasmid vectors e.g. the vectors from Vical, San Diego (Sedegah M, R Hedstrom, P Hobart, SL Hoffman, 1994. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. PNAS 91, 9866-9870) and the use of better delivery systems (to IM injection) employing a gene gun (Sun WH., Burkholder JK., Sun J., Culp J., Lu XG., Pugh TD., Ershler WB, Yang NS. IN VIVO CYTOKINE GENE TRANSFER BY GENE GUN REDUCES TUMOR GROWTH IN MICE. Proceedings of the National Academy of Sciences of the United States of America. 92:2889-2893, 1995.). In addition priming against CTL epitopes usually requires CD4 T cell help[17] thus the inclusion helper epitopes or proteins in the construct may improve the level and reliability of CTL priming by the murine DNA vaccine polytope.

71. **Lack of "Original antigenic sin" or the ability of a polytope to raise immune responses to all the epitopes in a polytope when the individual has already got a response to one of the epitopes.**

Introduction

72. Original antigenic sin is a term given to an antibody based phenomena whereby an existing antibody response to an epitope prevents the raising of an immune response to a second epitope when that epitope is present on the same protein as the first epitope (Benjamini E., Andria M.L., Estin C.D., Notron, F.L. & Leung C.Y. (1988) Studies on the clonality of the response to an epitope of a protein antigen. Randomness of activation of epitope -recognizing clones and the development of clonal dominance. *J. Immunol.* 141,55.). The reason for this phenomena is that large population of primed B cells specific for the first epitope bind and mop up all the available antigen before a naive B cell specific for the second antibody has a chance to bind the antigen, process it and present it to T helper cells. A similar situation might occur when an individual is vaccinated with a polytope when he/she already has a response to one of the

epitopes in the polytope. The existing CTL might kill all the polytope expressing cells before any of the other epitopes can be presented to naive T cells.

Method

73. To test this possibility mice (BALB/c) were infected with 10^4 pfu of Murine cytomegalovirus (MCMV) (K181 strain - Scalzo et al. 1995) and left for 5 weeks at which point strong CTL responses specific for the MCMV epitope, YPHFMPTNL, had developed (Scalzo et al. 1995 - Fig 2A). These mice were then given the murine polytope vaccinia and spleen cells assayed 10 days later for CTL specific for the three other epitopes presented by the polytope in this strain of mouse (RPQASGVYM, Lymphocytic choriomeningitis virus nuclear protein, H-2L^d; TYQRTRALV, influenza nuclear protein, H-2K^d and SYIPSAEKI, P. Berghei circumsporozoite protein, H-2K^d).

Sub D1

Results

74. Responses to each of the three new epitopes was observed following polytope vaccination, illustrating that the YPHFMPTNL specific CTL did not prevent priming of CTL specific for RPQASGVYM, TYQRTRALV and SYIPSAEKI when all four epitopes are presented together in the polytope. (Control animals receiving the human polytope vaccinia instead of the murine polytope vaccinia, showed only YPHFMPTNL specific CTL).

Sub D2

75. This series of experiments illustrate that if a polytope was, for instance, designed to cover a variety of different diseases, an individual receiving such polytope vaccine, but who had already been exposed to one of the target diseases would still be immunized against the remaining CTL epitopes in the polytope.

76. As will be apparent to those skilled in the art the present inventors have shown that the natural flanking sequences of CTL epitopes are not required for class I processing, that is each epitope within the polyepitope protein was always efficiently processed and presented to appropriate CTL clones by autologous polyepitope vaccinia infected target cells. It will be apparent to those skilled in the art that the polytopes may include sequences not naturally found to flank the epitopes.

77. As discussed above the present invention can be used with a range of epitopes. A range of epitopes are now available on an Internet address which is described in Brusic *et al* Nucleic Acids Research, 1994, 22; 3663-5.

78. It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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